

Potential of muscarinic and α -adrenergic responses by an analogue of guanosine 5'-triphosphate

(Ca^{2+} -dependent K^+ channels/ Ca^{2+} -dependent Cl^- channels/acetylcholine/inositol trisphosphate)

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ABSTRACT Ca^{2+} -dependent K^+ and Cl^- currents were recorded in isolated and dialyzed rat lacrimal gland cells by use of the tight-seal whole-cell recording technique. Under control conditions, application of acetylcholine (0.5–1.0 μM) resulted in the full activation of both types of current. When 50–200 μM guanosine 5'-[γ -thio]triphosphate (GTP[S], a nonhydrolyzable GTP analogue) was added to the intracellular solution, activation of both currents was seen with 1 nM acetylcholine, a dose 1/100th that needed under control conditions. Dialysis with solutions containing 200 μM GTP or cAMP had no, or only slight, potentiation effects. The effects of GTP[S] were obtained only when ATP was included in the intracellular solution. The potentiated responses to acetylcholine were blocked by increasing 10-fold the intracellular Ca^{2+} -buffering capacity and were not dependent on external Ca^{2+} . Thus, the potentiated responses appeared to result from a release of Ca^{2+} from internal stores. GTP[S] also greatly potentiated the Ca^{2+} -dependent adrenergic (norepinephrine) response of this preparation. In addition, GTP[S] elicited in some cells transient responses without application of acetylcholine or norepinephrine. Finally, rapid and sustained responses were seen as soon as the cells were dialyzed with inositol trisphosphate (20 μM). These findings are discussed in terms of a possible role of a GTP-binding protein as a link between activation of muscarinic or adrenergic receptors and initiation of Ca^{2+} release by inositol trisphosphate.

In exocrine glands, activation of muscarinic or α -adrenergic receptors leads to the liberation of Ca^{2+} from internal stores and subsequently to fluid and protein secretion (1, 2). Much recent work has centered on the role of inositol 1,4,5-trisphosphate (InsP_3), a product of the degradation of phosphatidylinositol 4,5-bisphosphate (PtdInsP_2), as one of the last links of the chain of events leading from the agonist-receptor binding to Ca^{2+} release (for review, see ref. 3). The initial molecular events in this pathway are, however, poorly understood. We therefore decided to study the effects of certain phosphorylated compounds which could be involved in the Ca^{2+} -release process.

Experiments were performed on cells isolated from rat lacrimal glands and dialyzed with the tight-seal whole-cell recording technique (4, 5). These cells have been shown to possess three classes of Ca^{2+} -dependent channels that are sequentially activated as the internal Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, progressively rises and that are selective for K^+ , Cl^- , and monovalent cations, respectively (6–9). Application of acetylcholine (AcCho) leads to Ca^{2+} release and to the opening of the Ca^{2+} -sensitive channels (7, 8). These effects are directly responsible for the fluid-secretion response (1, 8, 10). In the present work, measurement of Ca^{2+} -dependent

currents was used both as an index of this cellular response and as a method to detect $[\text{Ca}^{2+}]_i$ changes.

METHODS

Single cells were isolated from exorbital lacrimal glands of 5- to 7-week-old male Wistar rats by use of trypsin and collagenase (11). After isolation, cells were plated in culture dishes (Falcon 1008) containing Eagle's minimal essential medium and were kept in an incubator for up to 6 hr until use. Before the recordings, which were performed at room temperature (22–25°C), the incubation medium was changed to a solution containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , and 5 mM Hepes/NaOH (pH 7.2). Currents were measured using the tight-seal whole-cell recording technique. The standard intracellular solution contained 140 mM KCl, 2 mM MgCl_2 , 0.5 mM EGTA/KOH, 5 mM Hepes/KOH (pH 7.2), and 0.2 mM ATP. This EGTA concentration is high enough to keep $[\text{Ca}^{2+}]_i$ at a low level in the resting cell and is low enough to allow $[\text{Ca}^{2+}]_i$ to rise when the cell is stimulated with AcCho. Guanosine 5'-[γ -thio]triphosphate (GTP[S]) was purchased from Boehringer Mannheim. AcCho was applied externally by use of a fast micro-perfusion system (12) that had an exchange time at the onset of drug application of about 0.2 sec.

In order to follow the Ca^{2+} -dependent currents, the membrane potential was held at -60 mV and depolarizing voltage jumps of 60 mV were given. K^+ currents were measured at 0 mV, a potential where Cl^- and cation-selective channels were at equilibrium. Activation of the two other channel types was followed at -60 mV, a potential where K^+ currents are very small (8). As will be discussed below, due to the difference of Ca^{2+} -sensitivity between Cl^- -selective and monovalent cation-selective channels (8), only the former channel type contributed significantly to the inward current at -60 mV in the present experiments.

RESULTS

Potential of Muscarinic Responses. Typical responses to bath application of AcCho with and without GTP[S] are compared in Fig. 1. Responses obtained without the GTP analogue were as described before (7, 8). At low doses of agonist, the K^+ -current relaxation observed at 0 mV was increased (Fig. 1a). The minimum AcCho concentration needed to observe this K^+ -selective response was 50 nM or 100 nM, depending on the preparation. With higher concentrations of agonist, $[\text{Ca}^{2+}]_i$ rose to a high enough level to activate both K^+ currents (as measured at 0 mV) and inward

Abbreviations: AcCho, acetylcholine; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; GTP[S], guanosine 5'-[γ -thio]triphosphate; InsP_3 , inositol 1,4,5-trisphosphate; PtdInsP_2 , phosphatidylinositol 4,5-bisphosphate.

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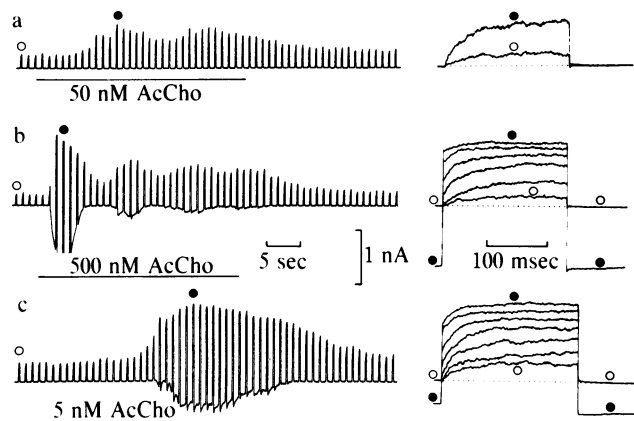


FIG. 1. Effect of AcCho application in control cells (*a* and *b*) and in a cell dialyzed with 200 μ M GTP[S] (*c*). (Left) Pen-recorder traces of cell-current responses to the application (bars below records) of 50 nM AcCho (*a*), 500 nM AcCho (*b*), and 5 nM AcCho (*c*). The cells were held at -60 mV. In these and in all other recordings shown, the resting current was indistinguishable from the 0 current level at the scale of the figure, and the resting resistance, measured by giving hyperpolarizing voltage pulses (not shown) was higher than 2 G Ω . Upward deflections show outward-current responses to depolarizing voltage pulses to 0 mV. (Right) Time-dependent currents recorded during the jumps indicated by open and filled circles in the low-speed records (Left). In each case, currents obtained before AcCho application (open circles) have been shown superimposed on currents obtained at the peak of the response (filled circles). In addition, relaxations at 0 mV are displayed in *b* and *c* during the fall of the AcCho-induced current, in order to show that K^+ currents have very similar kinetics in the two recordings. (The corresponding currents at -60 mV are not shown for the sake of clarity.) Leakage current and capacitive transients were subtracted.

Cl^- currents (as measured at -60 mV; Fig. 1*b*). Maximal activation of both channel types was achieved at 0.5–1 μ M AcCho. These currents are blocked by atropine (7, 8). The responses shown in Fig. 1 *a* and *b* were obtained with an intracellular solution containing 0.2 mM ATP. Essentially similar results were obtained in other experiments without ATP.

When GTP[S] (50–200 μ M) was included in the pipette solution in addition to ATP, large inward- and outward-current responses were obtained with 5 nM AcCho, a dose 1/10th the threshold concentration in the absence of the GTP analogue (Fig. 1*c*). Average values of the maximum current increase were 1.16 ± 0.40 nA (mean \pm SD, $n = 9$) and -0.40 ± 0.28 nA at 0 mV and at -60 mV, respectively, under the conditions of Fig. 1*c* (Table 1). The corresponding values obtained with 0.5 μ M AcCho and no internal GTP[S] (as in Fig. 1*b*) were 2.18 ± 0.64 nA at 0 mV ($n = 5$) and -1.54 ± 0.31 nA at -60 mV. A number of arguments indicated that the currents activated in the presence of GTP[S] were due to the Ca^{2+} -dependent K^+ channels (previously called BK channels; ref. 7) and Ca^{2+} -dependent Cl^- channels (8) involved in the control response. The outward currents at 0 mV were selectively blocked by adding to the test solution 2 mM tetraethylammonium, which is known to inhibit BK channels very effectively (7). The outward relaxations were similar in the presence or absence of GTP[S] and showed characteristic large amplitude fluctuations in both cases (compare Fig. 1 *b* and *c*). Likewise, the inward current observed at -60 mV had properties similar to those of the Ca^{2+} -dependent Cl^- channels previously described (8). In particular, inward tails with slow kinetics were observed when the potential was returned from 0 to -60 mV.

The results of Fig. 1 suggest that GTP[S] strongly potentiates the response to AcCho. Full K^+ and Cl^- responses were obtained at AcCho concentrations as low as 1 nM (but

not at 0.1 nM), whereas in the absence of GTP[S], an agonist concentration of at least 200 nM was needed to produce similar currents, and no response at all was observed at 20 nM. Thus, the GTP analogue lowered the effective agonist doses by a factor of at least 100.

Ca^{2+} -Dependent Currents in the Presence of GTP[S]. To test whether the Ca^{2+} -dependent K^+ and Cl^- channels were activated by a rise in $[Ca^{2+}]_i$, we increased 10-fold the Ca^{2+} -buffering capacity of the internal solution. We found that the response potentiated by GTP[S], like the control response (7, 8), was totally blocked under these conditions (Fig. 2*a*). This suggests that a rise in $[Ca^{2+}]_i$ was the trigger of the potentiated response. It was in fact possible to estimate the maximal level reached by $[Ca^{2+}]_i$ during AcCho application by comparing the characteristics of AcCho-induced currents with results of inside-out patch experiments (for BK currents: see refs. 8 and 9) and whole-cell recordings (for Cl^- currents: unpublished observations) at various levels of $[Ca^{2+}]_i$. Such comparisons, performed on both the K^+ and Cl^- response, indicated that the maximal level of $[Ca^{2+}]_i$ was between 0.1 and 1 μ M for a typical recording like that shown in Fig. 1*c*. [The resting $[Ca^{2+}]_i$, estimated by similar methods, is close to 10 nM (9)]. At this $[Ca^{2+}]_i$, monovalent cation-selective channels are not activated (unpublished results). Therefore, the inward current at -60 mV will be entirely attributed to Cl^- channels in the following.

To see whether the rise in $[Ca^{2+}]_i$ was due to any entry of Ca^{2+} from the bath solution or to a release from intracellular stores, we applied AcCho in a Ca^{2+} -free solution containing 0.5 mM EGTA (Fig. 2*b*). Since the potentiated response was still observed, it must have been due to Ca^{2+} release from intracellular stores, as has been previously shown for the control response (7, 8).

When ATP was omitted from the pipette solution, potentiating effects of GTP[S] were much less conspicuous (Table 1). An example is shown in Fig. 2*c*. When GTP[S] was replaced by GTP or cAMP, no or only slight potentiation effects were obtained (Table 1).

Some of the features of the responses recorded in the presence of GTP[S] differed from those of control responses

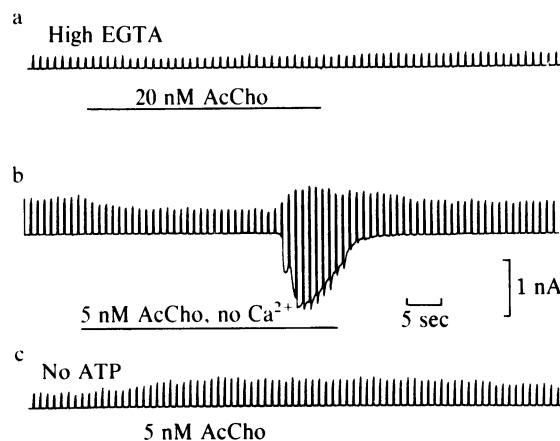


FIG. 2. Responses from three different cells, dialyzed with 0.2 mM GTP[S], to application of AcCho (shown by the bars below the records). (*a*) Lack of response to 20 nM AcCho when an internal solution with high Ca^{2+} -buffering capacity was used; the internal solution contained 140 mM KCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5.5 mM EGTA/KOH (calculated pCa 8), 5 mM HEPES/KOH, 0.2 mM ATP, and 0.2 mM GTP[S]. (*b*) Response to 5 nM AcCho applied in a Ca^{2+} -free solution containing 0.5 mM EGTA. Intracellular solution was as in *a*. The small decrease of K^+ currents occurring a few seconds after switching to the test solution was probably due to a reduction of $[Ca^{2+}]_i$ induced by the reduction in external Ca^{2+} . (*c*) Small response to 5 nM AcCho from a cell dialyzed with the normal intracellular solution containing 0.2 mM GTP[S], but without ATP.

Table 1. Effects of GTP[S], ATP, GTP, and cAMP on AcCho-induced currents

AcCho, nM	E_m , mV	Current, nA			
		GTP[S] + ATP	GTP[S], no ATP	GTP + ATP	cAMP + ATP
5	0	1.16 ± 0.40	$0.7, 0.3$	—	0
	-60	-0.40 ± 0.28	$-0.15, 0$	—	0
		(9)			(3)
20	0	1.87 ± 0.89	$0.1, 0$	0	0.56 ± 0.74
	-60	-0.55 ± 0.45	$-0.3, -0.1$	0	-0.06 ± 0.10
		(4)		(3)	(7)

The intracellular solution was supplemented with nucleotides as indicated (all nucleotides were used at 0.2 mM). Mean current and SD (in nA) and number of cells (in parentheses) are indicated; with the GTP[S], no ATP solution, only two experiments were performed both at 5 and at 20 nM AcCho, and their results are listed separately. For each preparation, results were obtained both with the reference solution, containing GTP[S] and ATP, and with one of the other solutions. This procedure minimized the effects of variations between cell preparations. No spontaneous activity comparable to that shown in Fig. 3 was observed with GTP[S] without ATP, with GTP plus ATP, or with cAMP plus ATP.

*Membrane potential.

to AcCho at comparable current levels. First, the potentiated response displayed a very long delay, typically 10–30 sec, whereas the control responses had a latency of about 1 sec (compare Fig. 1 *b* and *c*). Second, whereas AcCho-induced currents displayed damped oscillations in control experiments (Fig. 1*b*), a single “wave” lasting about 30 sec was observed in the presence of GTP[S] (Fig. 1*c*). (In certain cells, two successive waves were obtained with prolonged AcCho application, with a time interval of 35–80 sec between peak currents, compared to 5–10 sec under the conditions of Fig. 1*b*. These double waves suggest that the pseudo-period was increased at least 5- to 10-fold in the presence of GTP[S].) A third difference concerned the evolution of the response when AcCho was applied repeatedly. In the absence of GTP[S], AcCho-induced currents decreased markedly after a few applications (7), whereas in the presence of GTP[S], full AcCho responses could typically be elicited repetitively for about 30 min of recording, after which no responses could be obtained.

A final feature of the potentiated response was its reproducibility. Responses obtained in different cells with AcCho doses varying from 1 to 20 nM often had the same appearance, as if they reflected a fixed pattern of Ca^{2+} release. In fact, full responses sometimes occurred spontaneously without application of AcCho. Spontaneous responses (Fig. 3*b*) occurring between AcCho applications (Fig. 3 *a* and *c*) could conceivably have been due to contamination of the recording chamber by previous applications of AcCho. This was ruled out, however, since recordings obtained without AcCho in the microperfusion system also showed spontaneous responses (Fig. 3*d*).

α -Adrenergic Responses. In exocrine glands, activation of α -adrenergic receptors elicits Ca^{2+} -dependent permeability changes similar to those obtained with AcCho (13). Such a response can be obtained in isolated cells of rat lacrimal glands by measurements of labeled-ion fluxes (14). Under normal conditions, we found surprisingly weak responses to norepinephrine. Some preparations failed entirely to respond to concentrations up to 10 μM . In other cases, a weak increase of K^+ currents was obtained at 10 μM (Fig. 4*a*). This agonist concentration elicits maximal responses in nondialyzed cells (14). However, when the cell was dialyzed with GTP[S], a full response was obtained with norepinephrine concentrations ranging from 10 μM down to 100 nM (Fig. 4*b*). As previously shown for the muscarinic response, the norepinephrine-induced currents were abolished by a 10-fold

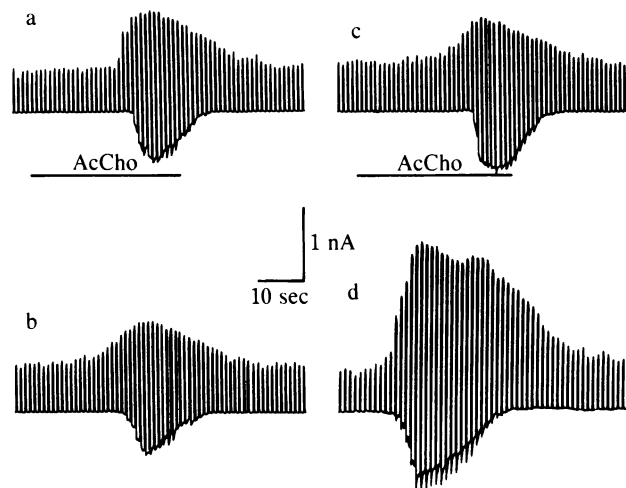


FIG. 3. Spontaneous waves of Ca^{2+} -dependent currents induced by GTP[S]. (*a–c*) Recordings from a cell dialyzed with 200 μM GTP[S], showing a spontaneous wave of Ca^{2+} -dependent currents (*b*) between two responses to 5 nM AcCho (*a* and *c*). Intervals between records were 41 sec (between *a* and *b*) and 81 sec (between *b* and *c*). (*d*) A spontaneous wave recorded in a cell that had not been exposed to AcCho. In this experiment, the microperfusion system did not contain AcCho.

increase in the Ca^{2+} -buffering capacity of the intracellular solution (Fig. 4*c*). The results of Fig. 4 show that GTP[S] restores a Ca^{2+} -dependent norepinephrine response which is present in nondialyzed cells (14). Qualitatively, this effect of GTP[S] resembles the potentiating action found with the muscarinic response.

The labeled-ion flux induced by adrenergic agonists is blocked by α -adrenergic antagonists such as phentolamine (14). In three experiments, norepinephrine (1 μM) responses obtained in cells dialyzed with GTP[S] (0.1 mM) were abolished if phentolamine (10 μM) was applied together with norepinephrine.

Effects of InsP_3 . Muscarinic and α -adrenergic stimulation result in increased cellular concentration of InsP_3 (3). InsP_3 is able to induce Ca^{2+} release from the endoplasmic reticulum of exocrine glands (15). In *Xenopus* oocytes, where muscarinic agonists evoke an electrical response similar to that of exocrine glands (16, 17), intracellular injection of InsP_3 was shown to mimic AcCho-induced currents (18). In lacrimal gland cells, addition of InsP_3 (20 μM) to the pipette

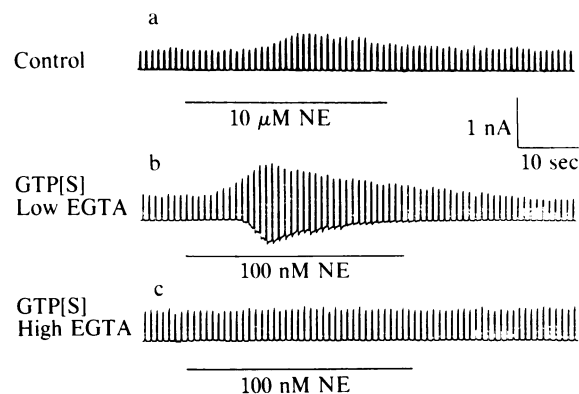


FIG. 4. Effect of GTP[S] on norepinephrine (NE) response. (*a*) Small response from a control cell when 10 μM norepinephrine was applied. (*b*) Application of 100 nM norepinephrine to a cell dialyzed with 200 nM GTP[S] results in a large K^+ - and Cl^- -current response. (*c*) Lack of response to 100 nM norepinephrine of a cell dialyzed with a high EGTA solution (that of Fig. 2*a*).

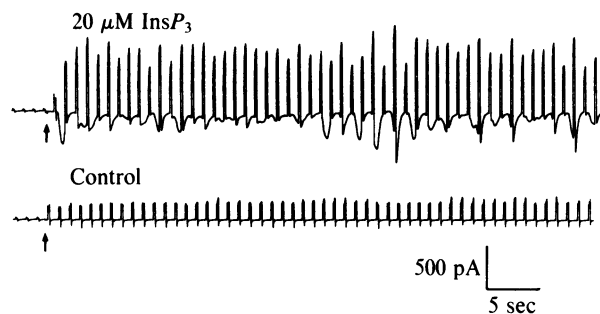


FIG. 5. Effects of InsP_3 . Recordings start in the cell-attached mode. As in other figures, repetitive voltage jumps from -60 to 0 mV were given throughout. Initiation of whole-cell recording (arrows) was indicated by an increase of the current noise, observable at high gain. Upper trace: pipette solution supplemented with InsP_3 (Sigma). [Other experiments, performed with InsP_3 purified from erythrocytes as described (19) and kindly provided by F. Giraud and M. Claret, gave very similar results.] Lower trace: control experiment (with another cell), with the pipette solution of Fig. 1 *a* and *b*. In the upper record, the first outward-current relaxation, 0.8 sec after the whole-cell configuration was established, has an amplitude comparable to that observed in the control. Inward current rose 1 sec after the whole-cell mode was established.

solution always resulted in a substantial increase of K^+ currents (which, however, did not reach saturation). In 6 cells out of 10, some activation of Cl^- currents was also obtained. Unlike the spontaneous activity evoked by GTP[S], the response was observed as soon as InsP_3 diffused into the cell, within 1 – 3 sec after the whole-cell configuration was established (Fig. 5; see ref. 5 for a discussion of the speed of diffusion exchange in whole-cell recording). The currents evoked by InsP_3 did not fade like those of Fig. 1*c*. Finally, they were observed in all cases, whereas the spontaneous activity associated with GTP[S] was obtained only in some of the cells. These findings suggest that InsP_3 is more directly linked to Ca^{2+} release than is GTP[S]. As in the case of the GTP[S]-potentiated response, it is possible to use previous inside-out and whole-cell recording results on K^+ and Cl^- currents to obtain a rough estimate of the Ca^{2+} level reached in the presence of InsP_3 . This gave a value oscillating between 0.1 and 0.5 μM in the experiment shown. In other cells, $[\text{Ca}^{2+}]_i$ rose to between 10 and 100 nM.

DISCUSSION

Our results show that internal dialysis of GTP[S] can mimic a functionally important muscarinic response—the opening of Ca^{2+} -dependent channels—and that it greatly potentiates the effects of muscarinic and α -adrenergic stimulation. The evidence from Fig. 2 suggests that it does so by acting on $[\text{Ca}^{2+}]_i$. The alternative view, according to which GTP[S] would somehow shift the Ca^{2+} -sensitivity of the channels, is unlikely since both K^+ - and Cl^- -selective Ca^{2+} -dependent channel types were affected.

It was recently proposed (20) that a GTP-binding protein (G protein), similar to those which stimulate or inhibit adenylate cyclase (proteins G_s or G_i , ref. 21) or to that which induces the degradation of cGMP in photoreceptors (transducin, ref. 22), was involved in the initiation of muscarinic responses. Recent reports (23, 24) that, in some preparations, GTP analogues induce the degradation of PtdInsP_2 to InsP_3 and diacylglycerol point to a possible role of a G protein in the regulation of cellular InsP_3 . Our results are entirely consistent with this proposal. Activation by AcCho and norepinephrine probably involves a receptor–G protein interaction similar to those occurring in the stimulation of G_s and G_i . In view of the striking similarities between potentiated AcCho and norepinephrine responses, it is tempting to speculate that

both neurotransmitters act via the same G protein. Since the adenylate cyclase controlled by G_s and G_i proteins produces cAMP, it is interesting that cAMP could not bypass the effects of GTP[S] or InsP_3 (Table 1). Thus, it is simplest to assume that the G protein directly induces the InsP_3 production. In order to explain that neurotransmitter-triggered and spontaneous responses observed in lacrimal gland cells last only for about 30 sec, it seems necessary to assume that in the present case, GTP[S] is slowly dissociating from its binding site. This contrasts with the situation found with G_s , G_i , or T proteins, where GTP[S] brings about an irreversible activation of the target enzyme (21, 22). An example of such an effect of GTP[S] in a cell dialyzed by the whole-cell recording method is the irreversible degranulation observed in mast cells when the GTP analogue was included in the dialysis solution (25). The hypothesis of a slow reversibility of GTP[S] binding also accounts for the observation that, in lacrimal gland cells, several responses to repeated agonist applications may be obtained in the presence of the GTP analogue. On the other hand, it was recently reported (26) that, when heart muscle cells are dialyzed with guanosine $5'$ - $[\beta, \gamma\text{-imido}]$ triphosphate, another nonhydrolyzable analogue of GTP, whole-cell current responses to AcCho become irreversible. In this respect, muscarinic responses in the heart differ from that of lacrimal glands.

An advantage of the experimental approach used in this paper is to allow the measurement of the time course of the cellular response to AcCho, InsP_3 , and GTP[S]. The quasiperiodic currents of Fig. 5 are reminiscent of the oscillations of tension that may be observed in skinned cardiac or skeletal muscle fibers (27), due to various feedback mechanisms regulating Ca^{2+} exchange between the cytosol and the interior of the sarcoplasmic reticulum. With AcCho (Fig. 1*a*) or with GTP[S] (Fig. 3), waves of activity are also observed, with pseudo-periods of 5 – 10 sec and 35 – 80 sec, respectively. These values are markedly larger than those observed with InsP_3 (1.7 sec in Fig. 5). This suggests that, in addition to Ca^{2+} -regulation systems of the endoplasmic reticulum, slower feedback mechanisms involving the G protein govern the production of InsP_3 . The type of approach used in the present work should help in understanding these regulatory processes.

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